

# Expression Cloning of *Lsc*, a Novel Oncogene with Structural Similarities to the Dbl Family of Guanine Nucleotide Exchange Factors\*

(Received for publication, November 16, 1995, and in revised form, March 29, 1996)

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**In a screen for genes with oncogenic potential expressed by the murine B6SUA<sub>1</sub> myeloid progenitor cell line, we isolated a 2.5-kilobase pair cDNA whose expression causes strong morphological transformation and deregulated proliferation of NIH 3T3 cells. The transforming cDNA encodes a truncated protein (designated *Lsc*) with a region of sequence similarity to the product of the *lbc* oncogene. This region includes the tandem Dbl homology and pleckstrin homology domains that are hallmarks of the Dbl-like proteins, a family of presumptive or demonstrated guanine nucleotide exchange factors that act on Rho family GTPases. *Lsc* requires intact Dbl homology and pleckstrin homology domains for its oncogenic activity. The transforming activity of *Lsc* in NIH 3T3 cells is reduced by cotransfection with p190 (a GTPase activating protein for Rho family GTPases) and the Rho family dominant-negative mutants RhoA(19N), CDC42(17N), and Rac1(17N). These results indicate a role for the Rho family of GTPases in mediating the transforming activity of *Lsc* and are consistent with the exchange specificities that have been attributed to Dbl family members. The *Lsc* gene is expressed in a variety of tissues and is particularly abundant in hemopoietic tissues (thymus, spleen, and bone marrow). *Lsc* is a member of a growing family of proteins that may function as activators of Rho family GTPases in a developmental or tissue-specific manner.**

The Ras superfamily of GTPases comprises approximately 50 members, many of which have regulatory functions in transducing intracellular signals. This superfamily can be conveniently divided into several families (Ras, Rho/Rac, Sar, Rab,

Arf, and Ran), the members of which are related in terms of both primary sequence and cellular activity (1–3). The Ras family itself is involved in triggering cell proliferation via activation of the Raf/MEK/mitogen-activated protein kinase signaling pathway (4–6). Activation of members of the more diverse Rho/Rac family has been correlated with changes in cytoskeletal organization (7–10) and with the transmission of growth factor and mitogen signals to the nucleus (11–14). The Rab, Arf, and Sar proteins regulate intracellular vesicle transport, while the Ran proteins function in nuclear transport (2, 3).

Ras-like proteins have a common intrinsic ability to bind and hydrolyze GTP. They exert their regulatory activity by acting as binary switches, cycling between an active GTP-bound form and an inactive GDP-bound form (1, 15). The rate of interconversion of Ras proteins between these two nucleotide-bound forms can be enzymatically modified by three families of regulatory molecules (2). GTPase-activating proteins (GAPs)<sup>1</sup> increase the intrinsic rate of hydrolysis of Ras-GTP to Ras-GDP, an irreversible reaction that inactivates Ras-like proteins. Guanine nucleotide dissociation inhibitors bind to and stabilize the GDP-bound form of Rho and Rab proteins, preventing their reactivation. Guanine nucleotide exchange factors (GEFs) accelerate the release of GDP and subsequent binding of GTP and thus serve as activators of Ras-like proteins.

GEFs have been described for virtually all the Ras GTPase families (2). One rapidly expanding group share a 250-amino acid stretch of significant sequence similarity with Dbl, a transforming protein that was originally isolated from a diffuse B cell lymphoma (16, 17). This domain (designated the Dbl homology or DH domain) was subsequently shown to contain GEF activity for the Rho family of GTPases (18). Many Dbl-related proteins, including Dbl itself, have been identified in transformation assays using NIH 3T3 cells as recipients (17, 19–23). DH domain-containing proteins that are truncated within this putative GEF catalytic domain consistently lose their transforming activity in NIH 3T3 assays (19, 20, 24). This association between DH domains, GEF activity, and transforming activity in NIH 3T3 cells implicates constitutive activation of Rho proteins as a general mechanism of transformation by Dbl-related proteins.

In addition to Dbl, the DH family consists of CDC24 (25), Vav (26, 27), Ect2 (23), Bcr (28), Abr (29, 30), RasGRF (31, 32), Tiam

\* This work was funded by grants from the Medical Research Council of Canada (to R. K.), the British Columbia Health Research Foundation (to R. K.), and the National Institutes of Health (to C. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U58203.

§ Supported by a fellowship from the Leukemia Research Foundation.

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<sup>1</sup> The abbreviations used are: GAP, GTPase-activating protein; kbp, kilobase pair(s); kb, kilobase(s); PCR, polymerase chain reaction; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; PH, pleckstrin homology; DH, Dbl homology; GEF, guanine nucleotide exchange factors; JNK, c-Jun N-terminal kinase; SRF, serum response factor.

(33), Tim (34), Lbc (22, 35), Ost (21), FGD1 (36), Dbs (20), and Lfc (19). CDC24, Dbl, Tiam, Ost and Lbc have been shown to have exchange activity toward Rho family GTPases (18, 21, 35, 37, 38). The Vav protein has been reported to stimulate exchange activity on Ras family members but shows no activity toward Rho/Rac GTPases (39–41). However, Vav appears to transform fibroblasts via Ras-independent mechanisms similar to those induced by Dbl (42, 43). Ect2 is able to bind to both Rho and Rac but does not exhibit exchange activity for either of these proteins (23). Although RasGRF contains a DH domain, it is also a member of a second structurally related family of GEFs that have activity toward Ras family members (2). The DH domain of RasGRF does not show exchange activity for either Ras or Rho family members (31). Two members of the Dbl family that have not been tested for GEF function, Bcr and Abr, have GAP activity toward Rho/Rac proteins (30, 44, 45), but this is mediated by a domain separate from their DH domains. The remaining members of the Dbl-related family (Dbs, Tim, FGD1, and Lfc) have not been assayed for any enzymatic activities.

In addition to a DH domain, Dbl family members possess a second shared domain, the pleckstrin homology (PH) domain. Since PH domains are found in a wide array of signaling molecules (46) and have been implicated in both protein-protein and protein-lipid interactions, it has been suggested that they may play a role in membrane translocation (47, 48). Dbl family members appear to have a requirement for intact PH domains to be transforming in NIH 3T3 assays (19, 20). Replacement of the PH domain of Lfc with a CAAX motif that directs membrane localization via prenylation completely restores its transforming ability (19), suggesting that PH domain-mediated membrane translocation may be a necessary step for transformation by Dbl family members in NIH 3T3 cells.

In studies aimed at identifying proteins from hemopoietic cell lines whose mis-expression can trigger deregulated cell growth, a cDNA library constructed from the murine B6SUA<sub>1</sub> myeloid progenitor cell line (49) was transferred to NIH 3T3 fibroblasts and screened for growth-deregulating activity, assayed by focus formation. In this study, we describe the isolation and characterization of a novel transforming protein, designated *Lsc*, that contains the tandem DH and PH domains that are characteristic of the Dbl family of exchange factors.

#### MATERIALS AND METHODS

**Cell Lines**—B6SUA<sub>1</sub> cells (49) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 5 ng/ml murine interleukin-3. NIH 3T3, C3H10T1/2, and C127 cells were obtained from American Type Culture Collection and cultured at low density in DMEM containing either 10% calf serum (NIH 3T3) or 10% fetal bovine serum (C3H10T1/2 and C127). The GP+E-86 (50) and BOSC 23 (51) packaging cell lines were cultured in DMEM containing 10% calf serum and 10% fetal calf serum, respectively.

**cDNA Library Construction**—mRNA was prepared from B6SUA<sub>1</sub> cells by lysis in guanidinium isothiocyanate as described (52), followed by binding to oligo(dT)-cellulose (53). cDNA was synthesized with random sequence hexamers and Moloney murine leukemia virus reverse transcriptase using methods provided by the supplier of the polymerase (Life Technologies, Inc.). *Bst*XI adapters (54) were added to the termini of the double-stranded cDNA, which was then ligated with *Bst*XI-cut pCTV3 retroviral vector (55). *Escherichia coli* MC1061/p3 (54) were transformed with the ligation mixture by electroporation and plated in soft agar (56). Pooled plasmid DNA was purified from the transformed bacteria by alkaline lysis (57), followed by digestion with RNase A and RNase T1, and precipitation with ethanol.

**Production of Viruses and Infection of NIH 3T3 Cells**—Library plasmid DNA was introduced into the GP+E-86 ecotropic packaging cell line by DEAE-dextran transfection (58). A 6-cm dish of cells at 70% confluence was washed twice with DMEM containing 20 mM HEPES, pH 7.2 (DMEM/H). Two ml of DMEM/H containing 2  $\mu$ g/ml plasmid DNA and 0.2 mg/ml DEAE-dextran ( $M_r$  500,000) was then added to the

cells, followed by a 1-h incubation at 37 °C in a humidified incubator with a 5% CO<sub>2</sub> atmosphere. The medium was then replaced with DMEM/H containing 10% calf serum and 0.2 mM chloroquine and the incubation was continued for another 3 h, after which the medium was replaced with chloroquine-free medium. At 48 h after the beginning of the transfection, the medium was replaced with DMEM/H containing 5 mM sodium butyrate (59). The medium was collected 24 h later and filtered. After the addition of an equal volume of fresh DMEM/H containing 10% calf serum and of Polybrene to a concentration of 10  $\mu$ g/ml, the medium was added to a 10-cm dish containing  $2 \times 10^5$  NIH 3T3 cells. The infecting medium was removed 18 h later, after which the NIH 3T3 cell culture was fed at 3-day intervals with DMEM/H, 10% calf serum.

Retroviral plasmids containing modified *Lsc* cDNAs were converted into retroviruses using the BOSC 23 ecotropic virus packaging cell line (51). All transfections were done in Falcon® six-well tissue culture plates (Becton-Dickinson). BOSC 23 cells were plated 24–48 h prior to transfection and were allowed to grow to 80% confluence in 2 ml of DMEM, 10% fetal calf serum. Two hundred microliters of Hepes-buffered saline solution (pH 7.05) was added to an equal volume of DNA (1–2 mg), CaCl<sub>2</sub> (25 mM) solution, and the resulting solution was immediately added to the above medium, followed by a 12-h incubation at 37 °C in a humidified incubator with a 5% CO<sub>2</sub> atmosphere. The medium was then replaced with DMEM containing 10% fetal calf serum, and the incubation was continued for another 12 h. The medium was collected 12 h later and filtered. After the addition of an equal volume of fresh DMEM containing 10% calf serum and of Polybrene to a concentration of 10  $\mu$ g/ml, the medium was added to six-well plates containing  $3 \times 10^4$  NIH 3T3 cells/well. The infecting medium was removed 12 h later, after which the NIH 3T3 cell culture was fed at 3-day intervals with DMEM, 10% calf serum.

**Recovery of cDNAs from Infected Cells**—Genomic DNA was prepared from infected NIH 3T3 cell clones by proteinase K digestion, phenol extraction, and ethanol precipitation. 25- $\mu$ l PCR reactions contained the following components: 20 mM Tris-Cl, pH 8.75, 10 mM KCl, 10 mM ammonium sulfate, 2 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 100 ng each of 5' and 3' primers, 300 ng of template genomic DNA, and 1.25 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Amplification was performed with the following thermal cycles: 95 °C for 60 s  $\times$  1; 95 °C for 60 s, 50 °C for 30 s, 72 °C for 180 s  $\times$  30. The amplified DNA was extracted with phenol:chloroform (1:1), ethanol-precipitated, and digested with *Mlu*I and *Bst*WI (restriction enzymes with recognition sites that flank the cDNA within the integrated provirus). The amplified cDNAs were purified by agarose gel electrophoresis, electro-eluted and ethanol-precipitated, and cloned by ligation into pCTV3 and transformation of *E. coli* MC1061/p3.

**Isolation of 5' Extended cDNAs**—Primers matching the 5' and 3' ends of the TL27-19-1-1 cDNA were synthesized and used in conjunction with primers matching sites in the vector that flank the ends of the cDNA insertion. PCR reactions were performed on plasmid DNA of the cDNA library, using *Pfu* DNA polymerase and the cycling parameters listed above. Amplified cDNA products were gel purified and cloned into pBluescript KS+ (Stratagene) for sequence determination. No discrepancies in sequence were found in the regions of overlap of the TL27-19-1-1 cDNA and the various PCR products. The complete *Lsc* cDNA (TL27-19-1-1 E1) was assembled by ligation of restriction enzyme-generated fragments of the TL27-19-1-1 cDNA and the A1 PCR product.

**Construction of TL27-19-1-1 Derivatives**—cDNAs encoding modified forms of the *Lsc* protein were generated by PCR amplification of the TL27-19-1-1 cDNA. Descriptions of termini are provided below. Numbers indicate nucleotide positions in the TL27-19-1-1 cDNA. D7: 5' = ACGCGTGTAA fused to 86; 3' = 2429; D4: 5' = ACGCGTCCCGG-GAACCACCATG fused to 1091; 3' = 2429; D3: 5' = 1, 3' = 2323 fused to TGATGAGGCGCC.

The D4 construct utilized an artificial start codon that had been introduced by PCR, while the D3 construct contained artificial stop codons.

PCR-directed mutagenesis was used to make the following amino acid replacement in TL27-19-1-1: M1, tryptophan (TGG) 2336–2338 to leucine (TTG).

All fragments that were synthesized by PCR were sequenced in their entirety to confirm that only specified mutations had occurred.

**Sequence Analysis and Comparisons**—cDNA sequences were determined by a chain termination procedure using thermal cycling with Vent thermostable polymerase (New England Biolabs). Continuous sequences were determined for both strands. Data base comparisons were performed with the MPSearch program, using the Blitz server operated

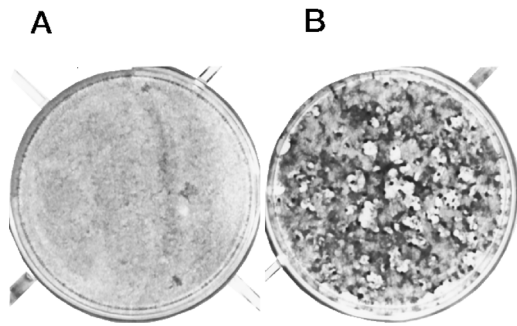


FIG. 1. **Transforming activity of the TL27-19-1-1 cDNA clone.** NIH 3T3 cells were infected at low density with CTV3 retroviral vector (A) or CTV3 carrying the TL27-19-1-1 cDNA (B). Cell cultures in 35-mm diameter wells were stained with methylene blue 5 days after reaching confluence.

by the European Molecular Biology Laboratory (Heidelberg, Germany). The sequence similarity analysis of *Lsc* and *Lbc* was performed with the Bestfit program of the Genetics Computer Group (Madison, WI).

**Focus Inhibition Assays**—The ability of p190 GAP and dominant negative mutants of Rac1, RhoA, and Cdc42 to modulate *Lsc* focus-forming activity was determined by cotransfection focus inhibition assays using procedures that have been previously described for Raf dominant negative proteins (60). Rac1(17N) and RhoA(19N) are dominant negative mutations of their respective GTPases that have been described previously (61). Wild-type p190 was kindly provided by R. Weinberg, while the dominant negative Cdc42(17N) mutant was provided by R. Cerione. DNA transfections were done as described previously using the calcium phosphate precipitation technique (62). Briefly, NIH 3T3 were transfected with 500 ng of pCTV3-*Lsc* retroviral expression vector alone, or together with 2 mg of pZip-p190, pZip-rhoA(19N), pZip-rac1(17N), or pCMV-cdc42(17N) expression vectors. The appearance of transformed foci was quantitated after 14–16 days. Transfected cultures were stained with 0.4% crystal violet to better visualize transformed foci. Relative focus-forming units were calculated based on the number of transformed foci present in control dishes that were transfected with empty vector alone. Data shown are representative of three or more independent determinations, with each determination representing the average number of foci from four dishes.

**Hybridization Analysis of RNA and DNA**—Total cellular RNA, mRNA, and genomic DNA were prepared as described above. RNA was separated by electrophoresis through agarose gels containing 0.66 M formaldehyde and transferred to Zeta-Probe nylon membranes (Bio-Rad). Hybridization and high stringency washing was performed as described (63), using DNA probes labeled with  $^{32}\text{P}$  by extension of random sequence primers (64).

## RESULTS

**Oncogenic Selection and Recovery of the TL27-19-1-1 cDNA Clone**—We have screened a cDNA library prepared from the B6SutA<sub>1</sub> myeloid progenitor cell line for clones that have transforming activity when expressed in NIH 3T3 fibroblasts. The B6SutA<sub>1</sub> library was constructed in the retroviral pCTV3 vector, converted into a library of viral clones by transient transfection of the ecotropic packaging cell line GP+E-86, and transferred to NIH 3T3 cells by infection. Numerous transformed foci arose when the cells reached confluence. One of these cell clones, TL27-19, was isolated, expanded, and examined by PCR for the presence of proviral inserts. Two provirus-derived fragments of 2.7 and 2.2 kbp were amplified, consisting of 2.4 and 1.9 kbp cDNAs, respectively, plus linked 300-bp *supF* genes. These fragments were purified and recovered by insertion into the retroviral vector pCTV3K using the *supF* gene as a selectable marker for *E. coli* transformation (55). The recovered cDNAs were converted to retroviral form and retested for transforming activity in three cell lines, NIH 3T3, C3H10T1/2, and C127. The NIH 3T3 cells infected with the 2.4-kbp cDNA (designated TL27-19-1-1) became very refractile within 3 days and continued to proliferate rapidly, forming large dense foci after confluence had been reached (Fig. 1). No transforming

activity was detected in the C3H10T1/2 or C127 cell lines or in any cell lines infected with the 1.9-kbp cDNA (data not shown).

**TL27-19-1-1 Encodes a Protein With Structural Similarities to the Dbl Family of GEFs**—The complete TL27-19-1-1 cDNA sequence has a length of 2429 bp with a single, long open reading frame starting with an ATG codon at nucleotide 92. This codon is in good context for translation initiation with a purine (G) at  $-3$  and a purine (G) at  $+4$  (65). Although there are no termination codons upstream of this site, the upstream sequence is GC-rich ( $>80\%$ ) and therefore is unlikely to be coding. PCR amplification of five cDNA libraries derived from cell lines in which TL27-19-1-1 is expressed failed to extend the cDNA at the 5' end. The open reading frame encoded by TL27-19-1-1 contains no termination codons indicating that the cDNA is truncated at its 3' end. Translational termination presumably occurs at a stop codon within the flanking *Bst*XI linker sequence. To obtain the sequence of the complete coding region (Fig. 2), a cDNA with an extended 3' end (designated A1) was isolated from an oligo(dT)-primed cDNA library derived from the GM979 erythroleukemic cell line. The 3' terminus of the A1 cDNA contains a poly(A) tract with a corresponding putative polyadenylation signal and probably represents the terminus of a full-length TL27-19-1-1 mRNA. The combined cDNA sequence (TL27-19-1-1 E1) has a length of 3200 bp and encodes a protein of 919 amino acids.

The TL27-19-1-1 cDNA has high sequence similarity (88% identity) to the HIBBk30 clone (66), a partially sequenced cDNA randomly isolated from a human infant brain cDNA library. The high degree of identity at the nucleotide level between these two clones argues that they are derived from homologous human and murine genes and that the translational products have retained a highly conserved function in these two species.

The protein encoded by the TL27-19-1-1 cDNA has structural similarities to the Dbl family of GEFs and in particular to *Lbc* and *Lfc*. *Lbc* is a Dbl family member isolated from a CML acute phase sample (22), while *Lfc* was isolated from the 32D murine myeloid cell line (19). The similarity between these three proteins precisely brackets the tandem DH and PH domains. Because it is more distantly related to *Lbc* than is *Lfc*, we have designated this new Dbl family member *Lsc* (*Lbc*'s second cousin). Over the 346-amino acid region of similarity between *Lbc* and *Lsc*, 31% of the residues are identical. Five gaps need to be inserted in the sequences to obtain this optimal alignment (Fig. 3). Over this same interval, *Lsc* and *Lfc* are 29% identical with 5 gaps inserted (data not shown). *Lsc* is distinguished from *Lbc* by an extended N terminus (412 amino acids), and although this region shows no significant similarities to known proteins, it does contain the highly conserved sequence match to the HIBBk30 human cDNA.

**Domains in the *Lsc* Protein Required for Transformation**—The *Lsc* cDNA that was recovered from the original transforming retrovirus is truncated downstream of the codon for Arg<sup>779</sup>. Thus, the protein encoded by this cDNA lacks a portion of the C terminus. NIH 3T3 cells infected with a CTV3 vector that expresses the full-length *Lsc* protein (TL27-19-1-1 E1; Fig. 4) become very refractile within 5 days and, subsequent to reaching confluence, continue to proliferate rapidly forming large dense foci. Since the transformed phenotype induced by the expression of the full-length TL27-19-1-1 E1 cDNA is morphologically indistinct from that induced by the originally isolated and 3' truncated TL27-19-1-1 cDNA, the C terminus of the *Lsc* protein is not required for, nor does it suppress, transforming activity. Similarly, the transformed phenotype induced by the expression of the TL27-19-1-1 cDNA (TL27-19-1-1 or D7 in Fig. 4) is morphologically indistinct from that induced by a modified

A C-terminal truncation of the Lsc cDNA that removes 13 of the C-terminal amino acids of the PH domain completely abol-

ishes transformation (Fig. 4). A point mutation in the conserved Trp<sup>749</sup> contained within this 13-amino acid region also abolishes the ability of *Lsc* to transform NIH 3T3 cells. Thus, as is the case with other Dbl family members, the PH domain of *Lsc* appears to be indispensable for transforming activity.

**p190 GAP and Dominant Negative Mutants of Rac1, RhoA, and Cdc42 Inhibit *Lsc* Transformation**—If *Lsc* is transforming through exchange activity for Rho family GTPases, we anticipated that blocking GTP-dependent activation of Rho family members should impair *Lsc*-mediated transformation. The p190 protein contains a C-terminal domain that has GAP activity for several Rho family GTPases (RhoA, RhoB, Rac1, Rac2, and Cdc42-Hs) in *in vitro* assays (67), and thus converts them to the inactive, GDP-bound form. Rac1(17N), RhoA(19N), and Cdc42(17N) are dominant-negative mutants of their respective rho family GTPases (61). The equivalent mutation in the Ras GTPase (Ras(17N)) has an enhanced affinity for GDP and is able to block signaling by endogenous proteins, possibly by titrating out an activating GEF (68, 69). Transformation of NIH 3T3 cells by expression of oncogenic Vav or Dbl is blocked when they are coexpressed with the Ras(17N) mutant (43). Rac1(17N) and Cdc42(17N) have been previously shown to be inhibitors of Rac1 and Cdc42 activity, respectively (70, 71).

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413 VKRQEVISELLVTEAAHVRMLRVLHDLFYQPMADGGFFPLDELQNIFFSLDE Lsc
    ||||| ||: || ||| :::: ||: | : | : :: :||:|
157 VKRQEVIELMQTEFHHVRTLKIMSGVYSQGMADLLFEQQMVEKLFPCLE Lbc

465 LIEVHSLFLDRLMKRRQES.....GYLIEEIGDVLLARFDGAEGSWFQKI Lsc
    ||: ||| :::: ||: || |||||: ||: |||: ||: ::: |
209 LISIHSQFFQRIERKKESLVDSKSEKFLIKRIGDVLVNOFSGENAEERLKK Lbc

510 SSRFCRSQSFALQKAKORRELRFCAVQEAESRPRCRLQLKDMIPTEMQ Lsc
    ::||: : :::: | : || ||| | ||| : : |
261 YGKFCGQHNQSVNYFKDLYAKDKRFQAFVKKKMSSSVVRLGIPECILLVTQ Lbc

562 RLTKYPLLLQSIGQNT.EESTERGKVELAAECREILHHVNOAVRDMEDLLR Lsc
    |:|:|:|:| | | :| : : : : : :| : | : |
313 RITKYPVLFQRILQCTKDNEVEQEDLAQSLSLVKDVIGAVDSKVASYEKKVR Lbc

613 LKDYQRRDLTN.LRESSDPMRELKNDITKKLVHEGPLTWRTKDKAIE Lsc
    | : : | : | : | : | : | :|:|:|:| : : : : |
365 LNEIYTKTDSKIMRMKSGQM...FAKEDLKRKKLVDRGVSFLKNAAGRLKE Lbc

664 VHVLLLDLQLLLQRODERLLKSHSRLTPTPDGKTLRPLRLTSAMTRE Lsc
    | : :| |:|:| | : : : | : | : | : | : |
414 VQAVLLTDILVFLQEKDQYIFASLDQKST.....VISLKKLIVRE Lbc

716 VATDHKAFYVIFTWQEAQIYELVAQTSSERKNWCNLITETAGSLKVP A Lsc
    || : |:::| : : : | : | | : | : | : | :
455 VAHEEKGLFLISMGMTPEMVEVHASSKEERNWIIQDIQTINTLSGNG Lbc

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FIG. 3. Comparison of the amino acid sequences of *Lsc* and *Lbc*. The sequences between residues 413 and 764 of *Lsc* and between 157 and 503 of *Lbc* were optimally aligned on the basis of residue identity (vertical lines) and similarity (colons).

NIH 3T3 cells were transfected with expression constructs of *Lsc*, either alone or together with p190, Rac1(17N), RhoA(19N), or Cdc42(17N) (Fig. 5). Whereas transfection with *Lsc* alone caused the appearance of  $\approx 65$  foci/ $\mu$ g of transfected DNA, p190 reduces the transforming activity by approximately 10-fold ( $\approx 4$  foci/ $\mu$ g). The dominant negative mutants RhoA(19N) and Cdc42(17N) reduced the transforming activity by 3–4-fold ( $\approx 20$  foci/ $\mu$ g), while dominant negative Rac1 reduced the transforming activity by 6-fold ( $\approx 10$  foci/ $\mu$ g). Expression of the wild-type GTPases had only slight suppressive effects on transformation by *Lsc*. The incomplete inhibition observed with the dominant negative mutations may be due in part to the incomplete transfer or expression of the dominant negatives in all the cells that acquire and express *Lsc*. However, it may also reflect an additive effect of activation of the different GTPases, as a combination of RhoA(19N) and Rac1(17N) suppressed transformation by *Lsc* somewhat more than Rac1(17N) alone. The inhibitory effect observed in the cotransfection with Rac1(17N) contrasts with the inability of Rac1(17N) to inhibit focus formation when cotransfected with RafCAAX, an engineered form of Raf kinase, which is constitutively targeted to the plasma membrane by the addition of a C-terminal prenylation signal (72).

**Expression of the *Lsc* Gene**—A variety of hemopoietic and non-hemopoietic cell lines were examined for expression of the *Lsc* gene (Fig. 6A). A probe derived from the full-length *Lsc* cDNA detected a major 3.0-kb mRNA in all cell lines, albeit with considerable variation in amount. This mRNA approximates the size of the TL27-19-1-1 E1 cDNA (3.2 kb, Fig. 2), providing further evidence that we have identified the major full-length *Lsc* cDNA. The 3.0-kb mRNA was present at relatively high levels in B6SutA<sub>1</sub>, the multipotent hemopoietic progenitor line from which the *Lsc* cDNA was derived. In addition, the *Lsc* probe detected less abundant mRNAs in B6SutA<sub>1</sub> at 4.6 and 9.0 kb. The major 3.0-kb transcript is expressed at high levels in a variety of hemopoietic cell lines and, to a lesser extent, in NIH 3T3 and C3H10T1/2 fibroblasts and the breast tumor C127 cell line.

In primary murine cells, the 3.0-kb *Lsc* mRNA was detected at relatively high levels in hemopoietic tissues (thymus, spleen, and bone marrow), at lower levels in kidney, at very low levels in stomach heart and skeletal muscle and is undetectable by Northern blot in brain or liver (Fig. 6B). In addition, this probe detected a less abundant 5-kb mRNA, in thymus and spleen.

**Structure of the *Lsc* Gene**—Restriction enzyme digestions of genomic DNA isolated from B6SutA<sub>1</sub> cells and normal mouse liver exhibited the same pattern of fragments hybridizing with a probe derived from the *Lsc* cDNA (Fig. 7). Therefore, the oncogenic B6SutA<sub>1</sub> cDNA does not appear to be derived from a gene that has undergone any gross rearrangements in the

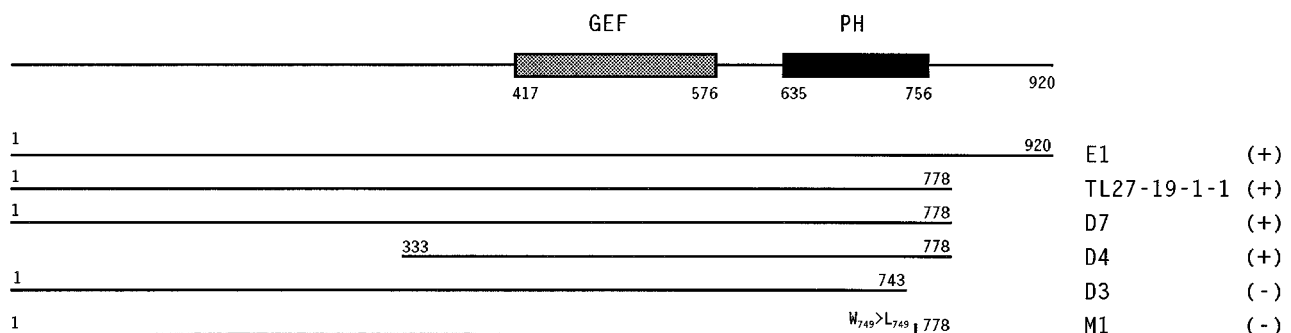
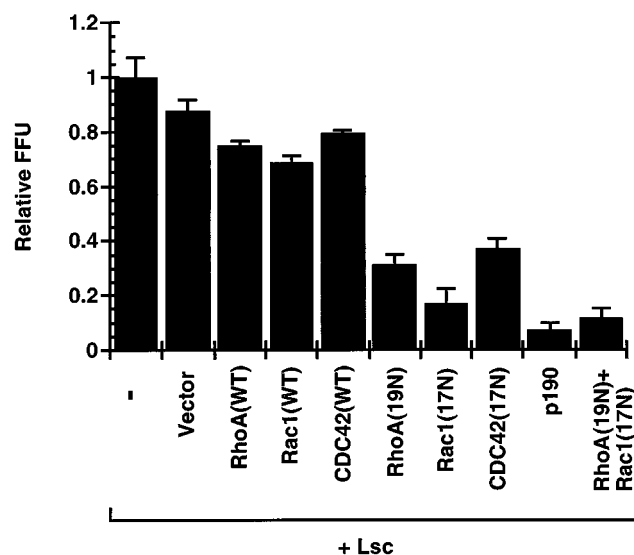


FIG. 4. Transforming activities of full-length, truncated, and mutated *Lsc* cDNAs. The domain structure of the TL27-19-1-1 encoded protein is illustrated in the upper line, and the lines below indicate the regions of the protein included in predicted translation products of the various truncated and mutated cDNAs. Boundaries of domains and predicted translation products are indicated with numbers referring to amino acid positions in the full-length protein as shown in Fig. 2. The symbols on the right indicate whether the cDNA had (+) or lacked (-) transforming activity when expressed in NIH 3T3 cells following their infection with retroviruses derived from the CTV3 vector carrying the indicated cDNA.



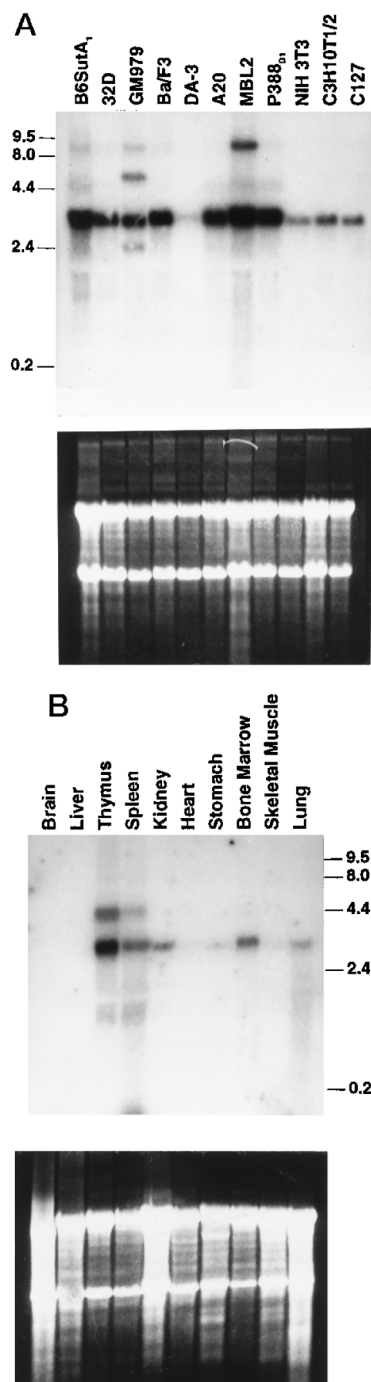
**FIG. 5. Suppression of *Lsc*-mediated transformation by p190 GAP or dominant negative forms of Rac1, RhoA, or Cdc42.** NIH 3T3 cells were transfected with expression constructs of *Lsc* alone or together with empty vector or vector carrying p190 Rho-GAP or the indicated wild-type or dominant negative GTPases. Foci were counted after 14–16 days. Relative focus-forming units were calculated based on the number of transformed foci present in control dishes that were transfected with empty pZip-NeoSV(x)1 retrovirus vector in the absence of *Lsc*. Data shown are representative of three or more independent determinations, with each determination representing the average number of foci from four dishes.

B6SutA<sub>1</sub> cell line. DNA fragments hybridizing to the *Lsc* probe were also observed in human genomic DNA, indicating that the *Lsc* gene has been conserved during the evolutionary divergence of mice and humans. This confirms the results of the data base comparisons, which indicate the existence of a human cDNA homolog.

#### DISCUSSION

We have identified a cDNA from the murine B6SutA<sub>1</sub> hemopoietic cell line that causes strong oncogenic transformation when expressed in NIH 3T3 fibroblasts. The novel protein that is encoded by this cDNA (designated *Lsc*) contains a domain that is characteristic of the Dbl family of GEFs. *Lsc* is most closely related to the Dbl family member Lbc, an exchange factor with specificity for Rho family GTPases (22, 35). *Lsc* is distinguished from Lbc by an extended N terminus (412 amino acids) that does not appear to be necessary for, or inhibitory to, cellular transformation.

The oncogenic transformation of NIH 3T3 cells that is induced by *Lsc* expression is likely to be a consequence of the activation of one or more Rho family members via *Lsc* GEF activity. Five members of the Dbl family (CDC24, Ost, Lbc, Dbl, and Tiam) exhibit *in vitro* exchange activity on Rho family GTPases (18, 21, 35, 37, 38) and activated mutants of RhoA and Rac1 can induce morphological transformation when expressed in NIH 3T3 cells (43, 61). In the present study, we observed that the p190 GAP and dominant inhibitory mutants of Rac1, RhoA, and Cdc42 blocked the transforming activity of *Lsc* in NIH 3T3 cells. These observations provide support for an *Lsc*-triggered activation of Rho signaling pathways and are consistent with the Rho family exchange activities that have been attributed to Dbl family GEFs. Using biochemical assays with purified Rho family GTPases, we have determined that *Lsc* promotes GDP-GTP exchange on RhoA, but not on Rac1 or



**FIG. 6. Expression of *Lsc* mRNAs.** RNA was separated by electrophoresis, transferred to nylon membranes, and hybridized with <sup>32</sup>P-labeled TL27-19-1-1 cDNA probes. Size markers are denatured DNA fragments of the indicated lengths that hybridize to the probe. Size markers are provided in kilobases. *Panel A*, total RNA from the indicated cell lines: 32D, GM979, DA-3, and B6SutA<sub>1</sub> (myeloid cell lines); Ba/F3 and A20 (B cell line); MBL2 (T cell line); P388<sub>D1</sub> (macrophage cell line); C3H10T1/2 and NIH 3T3 (fibroblast cell lines); and the C127 (breast tumor cell line). *Panel B*, total RNA from the indicated tissues. Ethidium bromide-stained gels are shown below the autoradiograms to show quantities of RNA.

Cdc42.<sup>2</sup> Together, these findings support a model in which RhoA is the immediate downstream effector of *Lsc* transformation, with activation of Rac and Cdc42 occurring subsequently, and contributing to transformation. Alternatively, *Lsc* may

<sup>2</sup> J. A. Glaven, I. P. Whitehead, T. Nomanbhoy, R. Kay, and R. Cerione, submitted for publication.



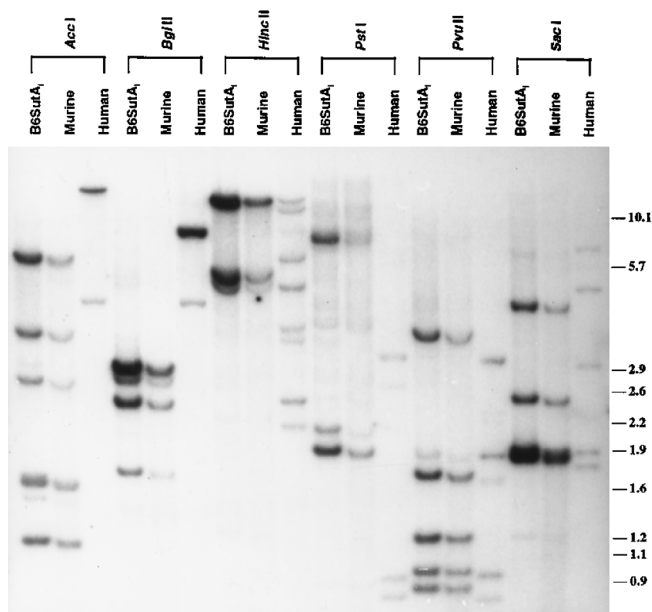


FIG. 7. **Structure of the *Lsc* gene.** DNA isolated from 32D cells, murine liver, or human peripheral blood leukocytes were digested with the indicated restriction enzymes, separated by electrophoresis, transferred to nylon membrane, and hybridized with the <sup>32</sup>P-labeled TL27-19-1-1 cDNA. Size markers are provided in kilobase pairs.

activate Rac or CDC42 independently of its function as a Rho-specific GEF, *e.g.* by binding to and relocalizing these GTPases, or other GEFs or GAPs.

Recent studies in yeast and mammalian cells have indicated that Rho family GTPases are tightly linked in regulatory circuits and that activation of a single GTPase (*e.g.* by a Rho family GEF) can trigger a cascade of GTPase activation (reviewed in Ref. 73). The assembly of actin-based cytoskeletal structures in mammalian cells involves a cascade of Rho family GTPases, in which Cdc42 activates Rac, which in turn activates Rho (8–10, 71). Recent studies suggest that activation of Rho family GTPases or their associated Dbl family GEFs can mediate the transmission of growth factor and mitogen signals to the nucleus via the c-Jun N-terminal kinases (JNKs; Refs. 11 and 12) through a mechanism distinct from that involved in cytoskeletal organization (11). In addition, RhoA, Rac1, and Cdc42 have been shown to regulate transcriptional activation by serum response factor (SRF; Ref. 13). There are several observations that suggest that the activation of these nuclear signaling pathways by Rho family GTPases does not involve the same GTPase cascade that is utilized during cytoskeletal reorganization. First, although SRF-linked signaling is activated by RhoA, Cdc42, and Rac1, Cdc42 and Rac1 signaling is not dependent on functional Rho (13); second, although Rac1 and Cdc42 are efficient activators of a cascade leading to JNK and p38/Mpk2 activation, RhoA does not exhibit this activity (12). If *Lsc*-mediated transformation is dependent upon multiple proliferative signals to the nucleus (*e.g.* through JNK and/or SRF), it may utilize several Rho family GTPases as immediate or secondary targets for its activity.

We have shown previously that the transforming activities of the Dbl family members Dbs and Lfc are dependent on the structural integrity of their respective PH domains. Disruption of these domains, either by truncation or by point mutations, completely abolishes transforming activity in NIH 3T3 cells (19, 20). We have also shown that the replacement of the PH domain of the Lfc protein with a CAAX motif that directs membrane localization via prenylation completely restores transforming activity, suggesting that a PH domain-dependent

recruitment of Dbl family members to the membrane may be a necessary step for transformation of NIH 3T3 cells (19). In the present study, we have observed that the transforming activity of the *Lsc* protein in NIH 3T3 cells is also dependent upon the structural integrity of its PH domain. This suggests that, like Lfc, *Lsc* may require membrane association for its transforming activity.

*Lsc* mRNAs were detected at relatively high levels in thymus, spleen, and bone marrow as well as a variety of hemopoietic cells, and at lower levels in some non-hemopoietic cell lines and tissues. Unlike the related Dbl family members, *lbc* and *lfc*, *Lsc* expression was not detected in brain tissue. The sizes of mRNAs hybridizing to *Lsc*-specific probes varied among cell lines and tissues. Although we have not determined whether the different mRNAs detected are all derived from the *Lsc* gene (*versus* very closely related genes), under the hybridization conditions used, such genes would have to be more closely related to *Lsc* than is *lbc*. It is more likely that the multiple mRNAs are due to differential splicing at the *Lsc* locus, and these could encode variant forms of the protein that in turn may confer some tissue specificity on the function of *Lsc*.

**Acknowledgments**—We thank Arthur Bank and David Baltimore for providing the GP+E-86 and BOSC 23 packaging cell lines, respectively. We thank R. Cerione for the Cdc42(17N) mutant cDNA and R. Weinberg for the p190 cDNA.

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